

The relationship between IL-17A and IL-22 expression and clinical severity in patients with moderate/severe persistent allergic rhinitis

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ABSTRACT

Purpose: Several reactions leading to numerous effects are regulated by IL-22.

However, the relationship between IL-22 and immunopathogenesis of allergic rhinitis (AR) has been rarely investigated. The aim of the present study was to investigate the levels of IL-22 and IL-17A in AR patients and their association with clinical severity of persistent allergic rhinitis (PAR).

Materials and methods: Thirty mild persistent allergic rhinitis (M PAR) patients, thirty moderate/severe persistent allergic rhinitis (M/S PAR) patients, and thirty healthy controls were enrolled in this study. Local production of IL-22 and IL-17A in PAR patients and healthy controls' nasal mucosa was examined by immunohistochemistry (IHC) and real-time polymerase chain reaction (RT-PCR) techniques. Serum levels of IL-22, IL-17A, specific immunoglobulin E (sIgE), and total IgE (tIgE) in PAR patients and healthy controls were determined by ELISA. In addition, blood eosinophil, nasal eosinophils per field, and total nasal syndrome score (TNSS) were also assessed.

Results: In comparison with healthy controls, production of IL-22 and IL-17A in M/S PAR patients increased significantly. Furthermore, serum levels as well as the mean number of IL-22⁺ and IL-17A⁺ cells in nasal mucosa correlated with sIgE, nasal eosinophil count, and TNSS.

Conclusion: The results of the present study provide the first evidence that local production of IL-22 might be expressed in PAR patients. The expression of IL-22 and IL-17A, and their correlations with clinical parameters in PAR patients suggest the role of these cytokines in the events involved in the development of PAR.

1. Introduction

Allergic rhinitis (AR) is a non-infectious disease caused by the contact of allergens during the presence of IgE in the nasal mucosa. A recently conducted study revealed that imbalance of Th17 and Treg cells and allergic rhinitis are highly correlated [1]. Furthermore, another recent study in this regard indicated that Th17 cells, which are classified as a subclass of TCD4⁺ cells, play a prominent role in the pathogenesis of allergic diseases and represent a new mechanism in the development of allergic rhinitis [2].

Th17 cells are recognized to produce a variety of cytokines such as IL-6, IL-17, TNFα, and IL-22. IL-17A is an inflammatory cytokine, which can affect a wide range of respiratory cells and can also induce the gene expression of cytokines and mucosal proteins [3,4].

Numerous experiments have addressed the role of IL-17A in pathogenesis of AR [5–7]. The results of studies targeting children

suffering from AR revealed that there was a significant positive correlation between IL-17A mRNA expression, which was caused by the presence of allergens inside the peripheral blood mononuclear cells (PBMC), and symptom medication score [5,8]. In addition, the levels of IL-17A in nasal lavage of patients with AR were significantly higher than those of healthy individuals in the control group [8]. Moreover, experiments using mouse models indicate that IL-17A is involved in both the development and regulation of AR [7,9,10].

Moreover, IL-22 can be regarded as an important factor in modulation of tissue response during the inflammation and carry out a prime function in inflammatory diseases [11]. IL-22 belongs to the family of IL-10 cytokines, and one of its most significant sources is TH17. IL-22 receptor is expressed over the surface of epithelial cells of respiratory system and can induce the expression of special antimicrobial peptides and proteins, which can cause cell dedifferentiation and survival [12–15]. Furthermore, an array of experiments proved that

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neutralization of IL-22 can increase the allergic response and migration of eosinophils into the lungs [16]. IL-22 regulates different processes and causes different effects. As the above-presented review of literature indicates, only few studies have been conducted on the local expression of IL-17A and IL-22 in patients with AR. Hence, the present study aimed at comparing the expressions of IL-17A and IL-22 in inferior turbinate mucosa of patients with mild and moderate/severe AR and those of healthy individuals. Therefore, the present study was undertaken to:

- 1) Compare the human serum expression levels of IL-17A and IL-22 in M/S AR patients, M AR patients, and healthy controls.
- 2) Evaluate the expression of IL-17A and IL-22 in human inferior turbinate mucosa and compare the expression levels in M/S AR patients, M AR patients, and healthy controls.
- 3) Specify any correlations between IL-17A and IL-22 expressions with various pathological parameters.

2. Material and methods

2.1. Participants

This study was conducted in the otolaryngology clinic of Kashani Hospital affiliated with Shahrekord University of Medical Sciences, Shahrekord, Iran. The present study was approved by the Ethics Committee of Shahrekord University of Medical Sciences (ETHICS COMMITTEE CERTIFICATE OF APPROVAL Numbered 92-7-23). Moreover, written informed consents were obtained from all participants. Sixty patients with previously demonstrated and not treated allergic Rhinitis, who had not received any drugs within the past four weeks, were chosen to participate in the study. Diagnosis of AR was based on the ARIA guideline (Allergic Rhinitis and its Impact on Asthma, 2008 update) [17]. The exclusion criteria were the presence of any inflammatory and autoimmune diseases and history of recurrent infections, chronic sinusitis and smoking. Patients who had previously used anti-allergy drugs, inhaled corticosteroids, or used systemic steroids within the past four weeks as well as those who were active smokers were excluded from the study. According to the results of prick test and presence or absence of clinical symptoms including sneezing, rhinorrhea, and nasal congestion over most days, the selected patients were divided into two groups consisting of mild persistent allergic rhinitis (M PAR) ($n = 30$) and moderate/severe allergic rhinitis (M/S PAR) ($n = 30$). Duration of the disease did not significantly differ between the groups of patients with M PAR and M/S PAR (Table 2). Moreover, thirty healthy, unrelated and age and gender matched control participants with no personal or family history of asthma, allergic rhinitis, and other inflammatory disease who had normal nasal mucosa and were admitted for augmentation rhinoplasty.

2.2. Prick test

Skin prick testing (SPT) was performed according to the guidelines of European Academy of Allergy and Clinical Immunology [18] and with consideration of the positive results (mean wheal diameter > 3 mm) specified for common aeroallergens.

2.3. Blood sampling and cytokine measurements by ELISA

Blood samples of patients suffering from house dust mite (Astra biotec, Germany) were obtained for assessment of eosinophil counts, serum tIgE (Pishtazteb, Iran), and sIgE using ELISA technique. Blood collection, handling, and storage were performed by the same researchers. Serum levels of IL-17A and IL-22 were determined by ELISA assays according to the manufacturer's instructions (Abnova, Taiwan). All assays were carried out in duplicate.

2.4. Hematoxylin and Eosin (H&E) staining and immunohistochemistry

During surgery in the otolaryngology clinic of Kashani Hospital, the inferior turbinate mucosa was obtained by a surgeon from the all patients and healthy individuals. Biopsy specimens were submerged immediately in 10% buffered formalin and prepared for paraffin embedding. Then, the paraffin blocks were cut into sections of four microns thick, which mounted on glass slides. They were prepared for routine H & E stain to perform the conventional histopathology and for immunohistochemistry to examine the presence of eosinophil in nasal mucosa in all groups. To perform the immunohistochemical examination of IL-17A and IL-22 proteins, the labeled streptavidin-biotin complex method and mouse and rabbit specific HRP plus (ABC) (Abcam, England) were used. From each participant, two sections were dried in a 70 °C oven overnight, deparaffinized using xylene, and then re-hydrated using a series of alcohols (100%, 100%, 80%, and 70%). The sections were then microwaved for 3 min with maximum temperature and then for 25 min with minimum temperature in citrate phosphate buffer (pH 6.0) for antigen retrieval. Blocking of the endogenous peroxidase activity was performed using 0.3% H₂O₂ in TBS for 15 min. The sections were then incubated with protein block (Abcam, England) for 10 min to block the non-specific background staining. Polyclonal anti-IL-17A and anti-IL-22 was generated by immunizing the rabbits (Abcam-England). 1 mg/ml of these antibodies used against IL-17A and IL-22 were applied as the respective primary antibodies, and tissue sections were incubated overnight at 4 °C. Afterward, the sections were washed with 1% bovine serum albumin (BSA) in Tris buffer solution (TBS). Then, biotinylated goat anti-rabbit and mouse IgG was applied, and the sections were incubated for 1 h at room temperature. Slides were developed with diaminobenzidine and counterstained with hematoxylin. Positive stain cells were calculated in five fields at the power of (10 × 10), where the highest cellular infiltration was noticed. The mean number of cells was then evaluated.

2.5. Quantitative PCR analysis

Total RNA was extracted from biopsies of the nasal inferior turbinate using Biozol reagent (Bioflux, Japan) according to the manufacturer's instructions. The quantity and quality of the RNA were measured by spectrophotometry at the wavelength absorption ratio of 260/280 nm. Some RNA samples (1 mg/ml) were coupled with DNase I (Thermo Scientific, Lithuania) for 30 min to avoid DNA contamination. Complementary DNA was synthesized using 1 mg of total RNA in a 20 ml reaction tube (Thermo Scientific, Lithuania) following the instructions provided by the manufacturer. PCR amplification reactions were conducted using a SYBR Green master mix (Takara, Japan). The reactions were incubated at 25 °C for 5 min, at 42 °C for 1 h, and at 72 °C for another 5 min. The primer sequences used for each gene, which was included in the study, are listed in Table 1. β -Actin was used as the internal control. All measurements were performed in duplicate. The levels of IL-17A and IL-22 mRNA expressions were assessed in relation to the housekeeping gene β -actin expression. Standard curves were prepared, and the efficiency was determined for each set of primers. The specified efficiency was typically above 90%. The data were expressed as a fold-change using comparative cycle threshold method

Table 1
Sequences of PCR primers.

| Primer | Sequence |
|------------|--|
| beta-Actin | Forward primer: 5-AGCCTCGCCTTTGCCGA-3 Reverse primer: 5-CTGGTGCCTGGGGCG-3 |
| IL-17A | Forward primer: 5-AATCTCCACCGCAATGAGGA-3 Reverse primer: 5-ACGTTCCCATCAGCGTTGA-3 |
| IL-22 | Forward primer: 5-GCAGGCTTGACAAGTCCAAC-3 Reverse primer: 5-GCCTCCTTAGCCAGCATGAA-3 |

($\Delta\Delta\text{Ct}$ method). The relative gene expression was determined by the $2^{-\Delta\Delta\text{Ct}}$ method (Table 1).

2.6. Assessment of IL-17A and IL-22 levels by ELISA

IL-17A and IL-22 levels in the normal, M PAR, and M/S PAR serums were measured using ELISA with the consideration of the manufacturer's protocol (Abnova, Taiwan). The data were expressed as pg/mg protein concentration. Protein concentration was determined using the ELISA Reader (Stat fax 2100, USA).

2.7. Statistical analysis

Statistical analyses were carried out using SPSS for windows (version 11, IBM SPSS statistics). In this study, Chi-square test was made use of to compare the differences of gender, congestion, rhinorrhea, and sneezing between the healthy controls and patients with M PAR and M/S PAR. A one-way ANOVA along with post-hoc Tukey test was performed to evaluate whether the values obtained by real-time PCR, IHC, and ELISA differ among the nasal mucosa of healthy controls and patients with M PAR and M/S PAR. Moreover, student's *t*-test was used to analyze the differences of sIgE between M PAR and M/S PAR patients. Correlations were assessed using the Pearson's correlation coefficient test. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Clinical characteristics of the participants

The characteristics of patients with allergic rhinitis and healthy controls are presented in Table 2. Age was similarly distributed among the healthy controls and the patients with allergic rhinitis. Duration of disease did not significantly differ between two groups of M PAR and M/S PAR patients (Table 2). In comparison with healthy individuals, serum tIgE levels were higher in patients with M PAR and M/S PAR (Table 3).

3.2. Gene expression levels of IL-17A and IL-22 in nasal mucosa

The gene expression levels of IL-17A and IL-22 were determined in healthy, M PAR, and M/S PAR nasal mucosa using real-time PCR. In comparison with healthy nasal mucosa, significantly increasing expression levels of IL-17A and IL-22 were observed in nasal mucosa of patients with M PAR and M/S PAR (Fig. 1A, B) (Table 3).

3.3. Local protein expression levels of IL-17A and IL-22 in nasal mucosa

The number of IL-17A⁺ and IL-22⁺ cells in nasal mucosa was significantly higher in M/S PAR group compared with that of the control group ($P < 0.05$; Fig. 2A, B). However, the difference between the number of IL-17A⁺ cells in control group and M PAR group was not

Table 2
Subject characteristics.

| Variable | Healthy controls | M PAR patients | M/S PAR patients |
|---|------------------|----------------|------------------|
| Gender (male/female) | 18/12 | 20/10 | 22/8 |
| Age | 28.5 ± 6.42 | 26.03 ± 4.03 | 26.7 ± 4.87 |
| sIgE for house dust mite (IU/mL) | 0 | 0.51 ± 0.15 | 4.57 ± 5.56 |
| tIgE (IU/mL) | 82.63 ± 43.88 | 236.3 ± 33.24 | 1061.1 ± 88.41 |
| Blood eosinophil counts | 74.5 ± 40.02 | 255.7 ± 70.96 | 607.27 ± 117.37 |
| Degree of eosinophil infiltration into nasal mucosa | 0.5 ± 0.5 | 1.7 ± 0.59 | 3.63 ± 1.88 |
| Disease duration | 0 | 15.63 ± 2.09 | 17.17 ± 4.39 |

Table 3

Comparison between the baseline characteristics, IL-17A gene expression, IL-22 gene expression, serum levels of IL-17A and IL-22, and IL-17A⁺ and IL-22 positive cells per field among healthy control, patients with M PAR, and patients with M/S PAR.

| Variable | Control | M PAR | M/S PAR |
|---------------------------------|---------------|-----------------------------|-----------------------------|
| IL-17A gene expression | 0.1267 ± 0.89 | 0.6103 ± 0.15 ^{ab} | 1.0570 ± 0.42 ^{bc} |
| IL-22 gene expression | 0.2525 ± 0.19 | 0.7277 ± 0.18 ^{ab} | 1.1527 ± 0.39 ^{bc} |
| Serum IL-17A level | 4.88 ± 2.34 | 18.55 ± 3.60 ^{ab} | 61.55 ± 18.49 ^{bc} |
| Serum IL-22 level | 14.55 ± 7.63 | 42.36 ± 11.86 ^{ab} | 80.81 ± 23.10 ^{bc} |
| IL-17A positive cells per field | 57.57 ± 5.41 | 62.70 ± 10.82 | 65.73 ± 6.79 ^{bc} |
| IL-22 positive cells per field | 68.30 ± 7.24 | 76.43 ± 12.64 ^{ab} | 83.07 ± 8.34 ^{bc} |

Results are shown as mean ± SD. A: a statistically significant difference between patients with mild persistent allergic rhinitis (M PAR) and healthy controls, B: a statistically significant difference between patients with mild persistent allergic rhinitis (M PAR) and patients with moderate/severe persistent allergic rhinitis (M/S PAR), and C: a statistically significant difference between healthy controls and patients with M/S PAR.

significant ($P < 0.05$). The difference between numbers of IL-22⁺ cells in M PAR and M/S PAR group was statistically significant ($P < 0.05$) (Table 3). Immunohistochemical localization of IL-17A⁺ and IL-22⁺ cells in nasal mucosa of healthy controls and patients with allergic was shown in Fig. 3.

3.4. Pathophysiological significance of IL-17A expression in nasal mucosa of patients with M/S PAR

A significant and positive correlation was observed between the number of IL-17A⁺ cells in the nasal mucosa and the total nasal symptom score (TNSS), which was determined considering the sum of sneezing, rhinorrhea, and congestion scores ($R = 0.680$; $P < 0.05$; data not shown). In details, the scores of congestion ($R = 0.476$; $P < 0.05$), rhinorrhea ($R = 0.377$; $P < 0.05$), and sneezing ($R = 0.508$; $P < 0.05$) positively correlated with the number of IL-17A⁺ cells. The degree of eosinophil infiltration into the nasal mucosa was highly and positively correlated with the number of IL-17A⁺ cells ($R = 0.564$; $P < 0.05$; data not shown). Moreover, there were significant correlations between IL-17A⁺ cells and sIgE ($R = 0.501$; $P < 0.05$; data not shown), serum IL-17A ($R = 443$; $P < 0.05$; data not shown), number of IL-22⁺ cells ($R = 0.508$; $P < 0.05$; data not shown), and tIgE ($R = 0.388$; $P < 0.05$; data not shown). However, the number of IL-17A⁺ cells did not statistically correlate with the blood eosinophil counts ($R = 0.311$; $P > 0.05$; data not shown).

3.5. Pathophysiological significance of IL-22 expression in nasal mucosa of patients with M/S PAR

There was a significant correlation between IL-22⁺ cells and TNSS ($R = 0.588$; $P < 0.05$; data not shown). In details, the associations between IL-22⁺ cells and congestion were noticeable ($R = 0.518$; $P < 0.05$); however, there were not any correlations between local production of IL-22 and rhinorrhea ($R = 0.33$; $P > 0.05$) and sneezing ($R = 0.305$; $P > 0.05$). Furthermore, the correlations between IL-22⁺ cells and the degree of eosinophil infiltration into the nasal mucosa ($R = 0.405$; $P < 0.05$; data not shown), sIgE ($R = 0.517$; $P < 0.05$; data not shown), serum IL-22 ($R = 0.776$; $P < 0.05$; data not shown), tIgE ($R = 0.37$; $P < 0.05$; data not shown), and blood eosinophil counts ($R = 0.427$; $P < 0.05$; data not shown) were statistically significant.

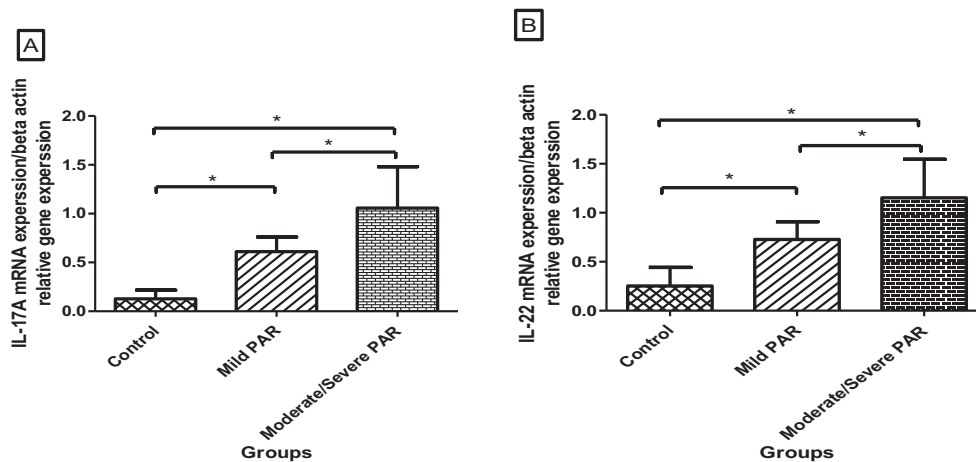


Fig. 1. Quantitative RT-PCR analysis of IL-17A (A) and IL-22 (B) mRNA in inferior turbinate nasal mucosal tissue from healthy controls and patients with mild persistent allergic rhinitis (M PAR) and moderate/severe persistent allergic rhinitis (M/S PAR). *Indicates significant difference in the expression levels of IL-17A and IL-22 among normal, M PAR, and M/S PAR nasal mucosa ($P < 0.05$). Results are shown as mean \pm SD.

3.6. Serum levels of IL-17A and IL-22 in healthy controls, patients with M/S PAR, and patients with M PAR

IL-17A and IL-22 levels (pg/ml) were measured in serum of the patients with M/S PAR, patients with M PAR, and healthy controls (Fig. 4A, B). In comparison with healthy participants ($P < 0.05$) and patients with M PAR, significantly higher levels of IL-17A and IL-22 were found in serum of patients with M/S PAR. Likewise, the differences between the serum levels of IL-17A and IL-22 in M PAR patients and healthy controls were statistically significant ($P < 0.05$) (Fig. 4) (Table 3).

3.7. Associations between the serum levels of IL-17A and the clinical characteristics and disease severity of patients with M/S PAR

There were significant correlations between serum levels of IL-17A and TNSS ($R = 0.654$; $P < 0.05$; data not shown), tIgE ($R = 0.575$; $P < 0.05$; data not shown), sIgE ($R = 0.561$; $P < 0.05$; data not shown), blood eosinophil counts ($R = 0.393$; $P < 0.05$; data not shown), and degree of eosinophil infiltration into nasal ($R = 0.405$; $P < 0.05$; data not shown).

3.8. Associations between the serum levels of IL-22 and the clinical characteristics and disease severity of patients with M/S PAR

Significant correlations were observed between serum levels of IL-22 and TNSS ($R = 0.716$; $P < 0.05$; data not shown), tIgE ($R = 0.342$; $P < 0.05$; data not shown), sIgE ($R = 0.568$; $P < 0.05$; data not shown), and the degree of eosinophil infiltration into the nasal mucosa

($R = 0.556$; $P < 0.05$; data not shown). However, the correlation between the serum levels of IL-22 and blood eosinophil counts ($R = 0.261$; $P > 0.05$; data not shown) was not statistically significant.

4. Discussion

In the present well-characterized cohort study, the exact cellular localization and expression patterns of IL-17A and IL-22 in human nasal mucosa of the inferior turbinate as well as the serum levels were described. Associations among expression of IL-17A, expression of IL-22, and immunopathogenesis of M/S PAR including disease severity and clinical characteristics, were also specified. To the best of researchers' knowledge, this study is the first one demonstrating the local production of IL-22 in nasal mucosa. The findings of the present study revealed that there were observable increases in the serum levels, gene expression, and local production of IL-22 in patients with M/S PAR compared with patients with M PAR and healthy controls. In line with the mentioned finding, in comparison with healthy individuals, serum levels of IL-22 have been observed to be up-regulated in patients with AR [19]. Likewise, serum levels and gene expression of IL-17A increased in patients with M/S PAR compared with M PAR and control groups. These results are in accordance with those obtained from other studies revealing the increased serum levels of IL-17A in patients with AR in comparison with healthy controls [8,20]. In contrast, the findings of another study indicated that the difference of serum levels of IL-17A between patients with AR and healthy controls was not statistically significant [21]. Interestingly, there were not any considerable differences in the local production of IL-17A in patients with M/S PAR and M PAR; in contrast, local production of IL-17A was significantly higher in

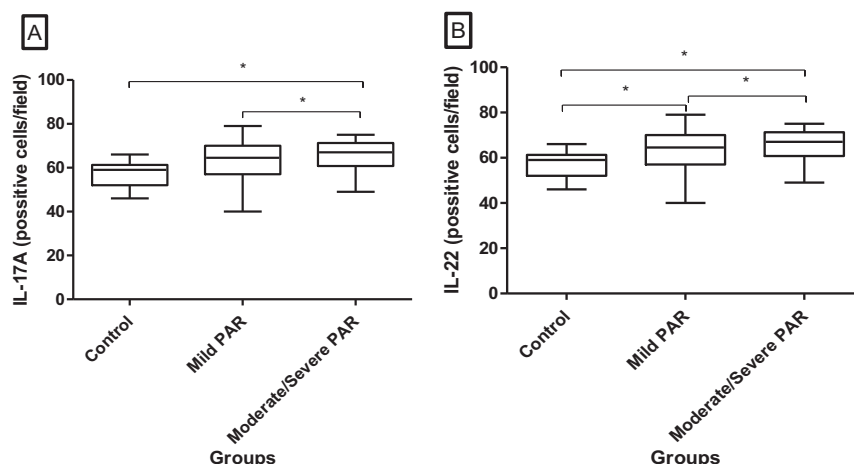


Fig. 2. Comparison between the number of IL-17A⁺ and IL-22⁺ cells in the nasal mucosa among healthy controls, patients with mild persistent allergic rhinitis (M PAR), and patients with moderate/severe persistent allergic rhinitis (M/S PAR). *Indicates significant difference in the expression levels of IL-17A and IL-22 among normal, M PAR, and M/S PAR nasal mucosa ($P < 0.05$). Results are shown as mean \pm SD.

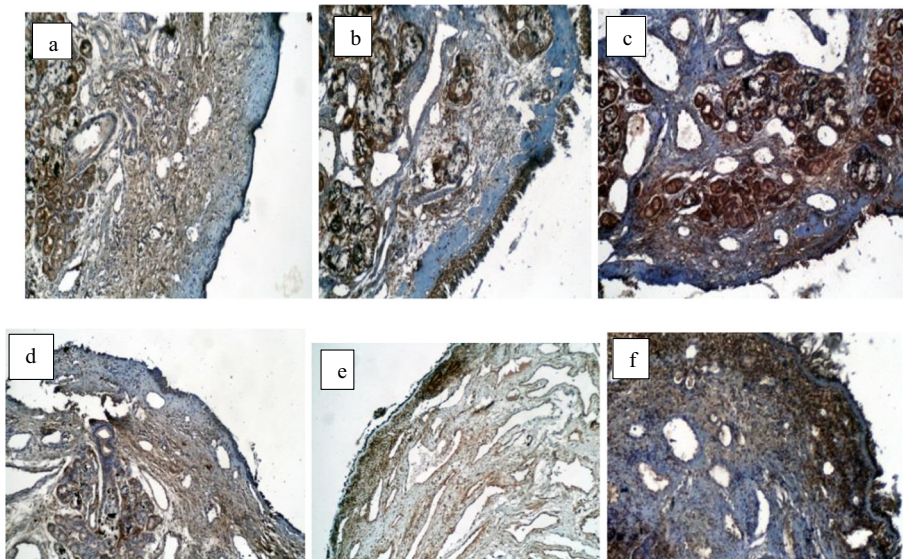


Fig. 3. Immunohistochemical localization of IL-17A (a, b, c) and IL-22 (d, e, f) in nasal mucosa of healthy controls (a, d), patients with mild persistent allergic rhinitis (M PAR) (b, c), and patients with moderate/severe persistent allergic rhinitis (M/S PAR) (c, f).

patients with M/S PAR in comparison with control group. The results of this study indicated that IL-17A⁺ cells, IL-22⁺ cells, and serum levels of IL-17A and IL-22 were highly and positively correlated with sIgE and the number of eosinophil infiltration into nasal mucosa. These results are confirmed by previous studies indicating the correlations among serum levels of IL-17A and the number of eosinophil infiltration into nasal mucosa [22] and sIgE [5,20]. Moreover, the association between serum levels of IL-22 and sIgE [19] indicated in the already conducted studies is in line with the findings of the present study. A limited chronic inflammation and the priming effect are stimulated by eosinophil infiltration into the nasal mucosa, both of which can intensify nasal hyperactivity [23]. Therefore, IL-17A and IL-22 may induce inflammation via enrichment of eosinophilic swelling. The present study demonstrated that serum levels of IL-22 did not associate with tIgE and blood eosinophil count; however, serum levels of IL-17A and IL-22⁺ cells were positively correlated with tIgE and blood eosinophil count. The findings of this study indicated that IL-17A⁺ cells associated with tIgE, although they indicated no correlations with blood eosinophil count. Makihara et al. [24] reported that IL-17A⁺ did not correlate with tIgE and blood eosinophil count. Similar to results of this study, Ciprandi et al.'s [8] study indicated that serum levels of IL-17A correlated with blood eosinophil count. Moreover, Farfariello et al. [25]

reported that serum levels of IL-22 did not correlate with tIgE. In this regard, more controversial results are presented addressing the correlations between IL-17A and TNSS. Ciprandi et al. [8] stated that serum levels of IL-17A were associated with TNSS in patients with AR, which was confirmed by the results of Nieminen et al.'s study [6]. Conversely, Lie et al. [26] and Klemenec et al. [27] reported that there were not any correlations between TNSS and serum levels of IL-17A in patients with AR. The current study showed that serum levels and local production of IL-22 and IL-17A statistically correlated with TNSS. The mentioned finding suggests that IL-17A and IL-22 are closely associated with the disease severity of M/S PAR.

In conclusion, this study provides the first report regarding the local production of IL-22 in inferior turbinate of nasal mucosa of patients suffering from PAR. Furthermore, the correlations between IL-22 and disease parameters are presented. Hence, these results strengthen the claim that IL-17A and IL-22 could be considered as potential markers of PAR severity.

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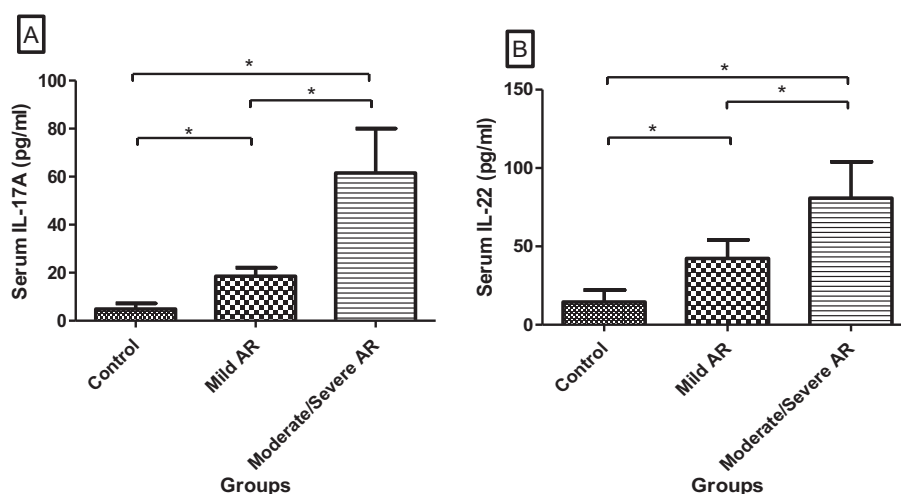


Fig. 4. Comparison between serum levels of IL-17A (A) and IL-22 (B) in healthy controls, patients with mild persistent allergic rhinitis (M PAR), and patients with moderate/severe persistent allergic rhinitis (M/S PAR). *Indicates significant difference in the expression levels of IL-17A and IL-22 among normal, M PAR, and M/S PAR nasal mucosa ($P < 0.05$). Results are shown as mean \pm SD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amjoto.2018.12.009>.

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